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Excretion of Nicotine and its Metabolites in Dog and Monkey Saliva[1]

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Excretion of Nicotine and its Metabolites in Dog and Monkey Saliva. TSUJIMOTO, AKIRA, KOJIMA, SEKIZO, IKEDA, MASAHIRO, and DOHI, TOSHIHIRO. (1972). *Toxicol. Appl. Pharmacol.* **22**, 365–374. Salivary and plasma levels of unchanged nicotine and total radioactivities (nicotine and its metabolites) were determined sequentially at short time intervals following iv injection of ^{3}H-labeled nicotine (100 μg/kg) to dogs and rhesus monkeys. Immediately after the drug injection, high concentrations of nicotine appeared in parotid and submandibular saliva induced by pilocarpine infusion or by auriculotemporal nerve stimulation. Excretory patterns of nicotine and total radioactivities in both glands were almost similar but were significantly different between the 2 species. Nicotine was excreted more readily in dog saliva than monkey. The salivary:plasma concentration ratios for nicotine were higher than those for total radioactivities in both species. Nicotine seems to be more readily excreted in saliva than its metabolites. The concentrations of nicotine and total radioactivities in saliva were independent of variation in salivary flow rate and were not affected by the method of stimulation. The chromatographic patterns of parotid and submandibular saliva resembled those of plasma in the same species. Chromatographic studies showed that most of the administered nicotine appeared in plasma and saliva in the form of cotinine 5–60 min after the injection. ^{3}H-Nicotine:total ^{3}H-radioactivity concentration ratios (^{3}H-nicotine:total ^{3}H) of both saliva and plasma in monkeys were lower than those in dogs. The times at which ^{3}H-nicotine:total ^{3}H ratio became 0.5 in plasma and saliva were very short (2–9 min); in addition, those in monkeys were short relative to dogs.

It has been reported that nicotine induces salivary secretion (Larson *et al.*, 1961) and that a high level of nicotine and its metabolites persist longer in the salivary glands than in other tissues (Hansson and Schmiterlöw, 1962; Yamamoto *et al.*, 1968). On the other hand, it has been demonstrated that the rhesus monkey was 5–10 times less sensitive than the dog to the effects of nicotine on gastrointestinal contractility (Hug and Bass, 1970). The rates of nicotine excretion by way of the kidney and the nature of the metabolites of nicotine have been studied in the dog (Larson *et al.*, 1961; Larson and Silvette, 1968).

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Salivary excretion of drugs and other substances foreign to the body is well documented. There has been no report on the salivary route of nicotine excretion. In studies on the salivary route of drug excretion, samples can be obtained sequentially at short time intervals.

The present report describes kinetic studies on excretion of nicotine and its metabolites by the salivary route compared to plasma levels in dogs and monkeys after iv injection of small doses of nicotine.

METHODS

Radioactive nicotine. ^{3}H-labeled nicotine (324–390 mCi/mole) was obtained from Radiochemical Centre, Amersham. The compound was identical to authentic, nonradioactive, redistilled nicotine in its absorbancy spectra (λ_{max} 259 nm) and its R_f value on paper chromatograms. The labile tritium was removed by extraction of the ^{3}H-nicotine from an alkaline aqueous solution in the *n*-hexane (as described below). The ^{3}H-nicotine was recovered in aqueous solution by extraction of the hexane with 0.1 N HCl solution.

The ^{3}H-nicotine was diluted with nonradioactive nicotine to provide a total dose of about 10 μCi of ^{3}H per kg of body weight, and in some cases in which chromatographic studies were carried out, the ^{3}H-nicotine was diluted to provide about 80 μCi of ^{3}H per kg of body weight.

Estimation of radioactive nicotine. ^{3}H-nicotine was estimated by the methods of Hug (1970). Aliquots of 0.1–0.2 ml of plasma and saliva were transferred to glass stoppered 50 ml centrifuge tubes containing 0.2 ml of nonradioactive nicotine base (2.5 mg/ml). The pH was adjusted to over 9 with a few drops of ammonium hydroxide reagent to which 1 ml of 40% K_3PO_4 was added followed by 10 ml of *n*-hexane. The mixture was shaken for 30 min at 280–300 oscillations per min and centrifuged at 3000 rpm for 5 min. From each sample, 8 ml of the organic phase was transferred to 20 ml scintillation-counting vials, and 8 ml of a toluene phosphor solution (containing 6 g of 2,5-diphenyloxazole (PPO) and 200 mg of 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP) dissolved in 1 l of analytical grade toluene) was added. Samples were counted in a liquid scintillation spectrometer (Packard Model 3320 or Aloka LSC-601). Recoveries of 50–1000 ng of ^{3}H-nicotine added to plasma averaged 99 ± 0.9% (SE, $n = 13$) and added to saliva were 97 ± 1.3% (SE, $n = 14$).

Estimation of total radioactivity. Aliquots of 0.05–0.1 ml of saliva were transferred to scintillation-counting vials; then 10 ml of a toluene phosphor solution containing ethanol (mixture of 3 volumes of ethanol and 7 volumes of the toluene phosphor solution) was added. Aliquots of 0.1 ml plasma were transferred to scintillation-counting vials containing 0.5 ml of glass distilled water. Then 10 ml of Instagel (obtained from Packard Instrument Co.) was added. Readings of cpm were converted to dpm by external standardization.

Chromatographic studies of samples containing nicotine. The sample was applied to Whatman No. 1 chromatographic paper along with authentic nicotine and cotinine which were diluted with saliva or water as standards; 1 ml of plasma samples was deproteinized by the addition of 0.19 ml of 30% trichloroacetic acid and adjusted to pH 7 with 1 N NaOH. Chromatograms were developed in either the "acid system"

(*sec*-butanol–90% formic acid–glass distilled water 7 : 1 : 1 ... tem" (0.5 N ammonia water–95% ethanol–*n*-butanol 1 : 1 : 1.4 v/v) using th... descending method at room temperature (18–25°C) in the dark (McKennis *et al.*, 19...

Authentic compounds were localized on dry chromatograms by visualization by spraying with a 2% w/v solution of *p*-aminobenzoic acid in ethanol followed by exposure of the chromatograms to cyanogen bromide vapor (McKennis ... *al.*, 1962). The R_f value was calculated for the most central point of the colored ...

Scanning chromatograms for radioactivity involved cuttin... the path of migration (2.2 cm in width) and dividing it lengthwise into 1 cm sectio... each section was placed in a scintillation counting vial; 10 ml of the toluene pho... solution containing ethanol was added, and the radioactivity was determined. The average cpm were plotted against cm of migration from the origin. The R_f value for an area of radioactivity was calculated using the centimeter section containing the highest cpm.

Animal experiments. Dogs, 7–12 kg, and rhesus monkeys of either sex, 3–4 kg, were anesthetized by ip injection of pentobarbital (30 mg/kg). Subsequent small doses of pentobarbital were given in order to maintain the anesthesia when necessary. The trachea was exposed and cannulated. Two parotid and 2 submandibular ducts were cannulated using polyethylene tubing. Salivary secretion was evoked by iv injection of 100 μg/kg of pilocarpine followed by infusion of 200–400 μg/kg/... min. In some cases, the peripheral cut end of the auriculotemporal nerve was electri... stimulated using bipolar platinum electrodes with submaximal shocks of 2 msec du... ...10 Hz. The saliva secreted for every 1 min was collected separately in a test tube. Blood samples were obtained through polyethylene tubing inserted into the femoral artery. The first several drops of each blood sample were discarded since it represented stagnant blood from the tubing. The blood was placed in tubes containing heparin, mixed gently ... avoid hemolysis and centrifuged.

When the salivary flow reached a steady level, radioactive nicotine was injected iv via polyethylene tubing which was inserted into the femoral vein for 10 sec. It was followed by infusion of 2–3 ml of saline. Zero time was marked at the point of completion of the nicotine injection.

RESULTS

Salivary Secretion Produced by Pilocarpine

After iv injection of 100 μg/kg of pilocarpine, prominent salivary flow from dog salivary glands was observed. About 5 min after beginning of the infusion of 200 μg/kg/60 min of pilocarpine, the salivary flow reached a steady level. Dog submandibular salivary flow was 15–25 drops (380–650 μl) per min, and the parotid salivary flow was 12–20 drops (300–500 μl) per min. The dog submandibular salivary flow was a little higher than the parotid salivary flow.

Monkey salivary glands were found to be about 2 times less sensitive to pilocarpine than dog salivary glands; viz. 20 μg/kg produced obvious salivary flow in dogs, but in monkey 40 μg/kg was needed to produce obvious salivary flow. The doses of pilocarpine infused were increased to 400 μg/kg/60 min in monkeys. Under the infusion of pilocarpine, monkey submandibular salivary flow was 4–6 drops (100–150 μl) per min, and the parotid flow was 6–8 drops (150–200 μl) per min. The monkey submandibular salivary flow was lower than the parotid.

In dogs and monkeys, the iv injection of 100 μg/kg of nicotine caused a slight acceleration in the submandibular and parotid secretion induced by the continuous infusion of pilocarpine, which was followed by a depression of 10–30% of control level for 1 to ca. 4 min and then recovered to the control level in about 10 min. Nicotine also first augmented and then depressed the dog parotid secretion induced by auriculotemporal nerve stimulation.

Excretion of Nicotine in Dogs

The levels of unchanged ^{3}H-nicotine and total ^{3}H-radioactivities in saliva after iv injection of 100 μg/kg of ^{3}H-nicotine to dogs are shown in Fig. 1. The concentrations of nicotine and total radioactivities in the submandibular saliva were highest for a 1–2 min

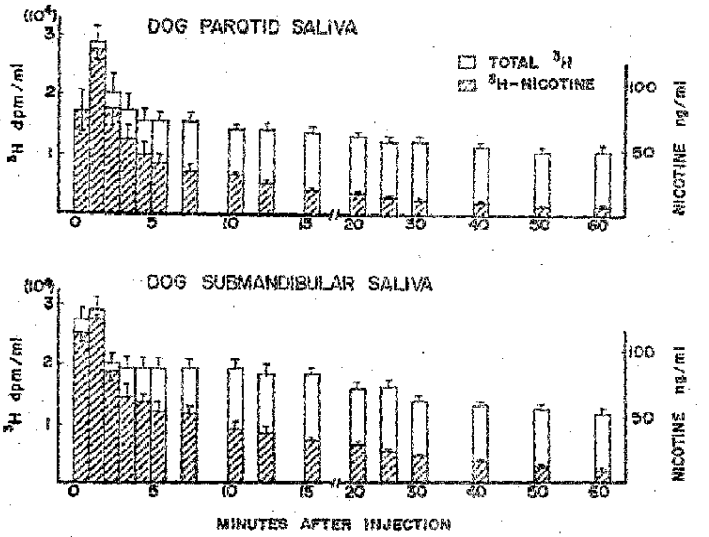

FIG. 1. Levels of ^{3}H-nicotine and of total ^{3}H-radioactivity in dog parotid and submandibular saliva following iv injection of ^{3}H-nicotine (100 μg/kg). Each bar represents the mean ± SE of 6 experimental values.

period after the nicotine administration. After that, concentrations of nicotine were rapidly reduced with time up to 4 min and then reduced slowly. The nicotine excretion pattern in the parotid glands was similar to that in the submandibular glands. The concentrations of nicotine and total radioactivities in the parotid saliva, however, were lower than those in the submandibular saliva. The concentrations in the parotid saliva from 0 to 1 min were 70% of those in the submandibular saliva.

The relationship between salivary flow rate and excretion of unchanged nicotine and total radioactivities in dogs was examined. Salivary flow rate was changed over a range of ca. 100–1200 μl/min by alteration of pilocarpine doses infused or by varying conditions of electrical stimulation of auriculotemporal nerve. After changing the flow rate, the first 10 drops of saliva were discarded to rule out the influence of the rate change transient proposed by Burgen (1964). The salivary concentrations of nicotine and total radioactivities were not affected by salivary flow rate. There was little difference in concentrations of nicotine and total radioactivities between the pilocarpine-induced saliva and the saliva secreted by electrical stimulation of auriculotemporal nerve.

The cumulative percentages of the total administered dose recovered in parotid and submandibular saliva for the first 1 hr after the injection averaged 0.30 ± 0.06 (±SE) as total radioactivity, 0.06 ± 0.01 (±SE) as unchanged nicotine, and 0.26 ± 0.01 (±SE) as total radioactivity and 0.19 ± 0.04 as nicotine, respectively.

Excretion of Nicotine in Monkeys

The highest concentration of nicotine in the submandibular saliva was observed during a 1–2 min period. After this period, the concentrations of nicotine were rapidly reduced with time up to 10 min and then reduced slowly (Fig. 2). Total ^{3}H-radioactivities were increased with time up to 5 min and then reduced very slowly. The nicotine excretion pattern in the parotid glands was similar to that in the submandibular glands.

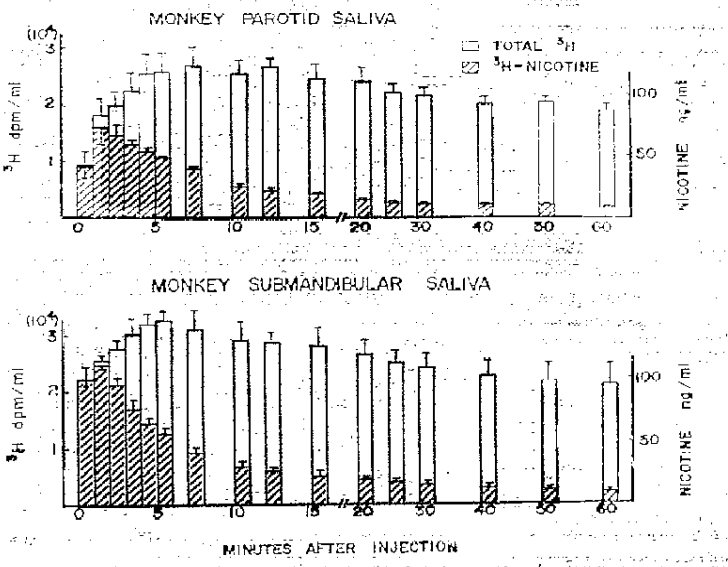

FIG. 2. Levels of ^{3}H-nicotine and of total ^{3}H-radioactivity in monkey parotid and submandibular saliva following iv injection of ^{3}H-nicotine (100 μg/kg). Each bar represents the mean ± SE of 3 experimental values.

Concentrations of nicotine and total radioactivities in parotid saliva, however, were low relative to those in submandibular saliva. The concentrations in the parotid saliva from 0 to 1 min were 40% of those in the submandibular saliva.

The cumulative percentages of the total administered dose recovered in parotid and submandibular saliva for the first 1 hr after the injection averaged 0.30 ± 0.06 as total radioactivity, 0.07 ± 0.01 as nicotine, and 0.26 ± 0.01 as total radioactivity, 0.07 ± 0.01 as nicotine, respectively.

Nicotine Concentration in Saliva and in Plasma

The nicotine concentrations in dog and monkey parotid saliva were lower than those in plasma during the period observed after the nicotine administration. Submandibular saliva in dogs had much higher concentrations of nicotine than those in plasma, while in monkeys it had lower concentrations during the first 30 min [illegible]

and had slightly higher concentrations at the later time [illegible]. The contents of total radioactivities in the parotid and submandibular saliva were low relative to those in plasma in both species during the period observed.

The salivary:plasma concentration ratios (S:P ratios) for nicotine and total radioactivities in dogs and monkeys [illegible] presented in Fig. 3. The time course of S:P ratios for total radioactivities was similar in both glands, but was significantly different between the 2 species. At earlier times (5 min), the S:P ratios for total radioactivities were about 1 in dogs but were much lower in monkeys (0.33 for parotid saliva and 0.48 for submandibular saliva). After that, the S:P ratios for total radioactivities in monkey rapidly increased with time up to 7.5 min and then increased to about 0.9; those in dog rapidly reduced with time up to 7.5 min and then reduced to about 0.6.

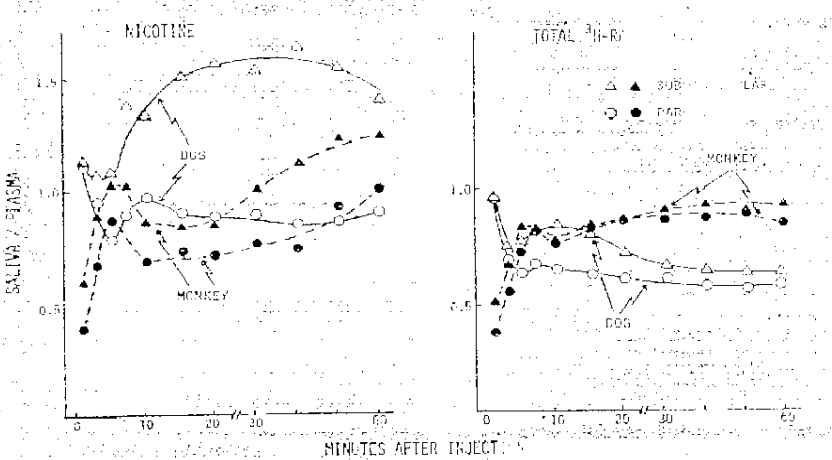

FIG. 3. Salivary:plasma concentration ratios for ³H-nicotine [illegible] and monkeys following the injection of ³H-nicotine. The [illegible] animals as those in Figs. 1 and 2.

A similar tendency could be observed in the time course of S:P ratios for nicotine and the S:P ratios for nicotine were significantly higher as compared to those for total radioactivities of both glands of dogs during the period observed. The S:P ratios for nicotine in monkeys also were higher than those for total radioactivities except for a few points.

The unchanged ³H-nicotine:total ³H-radioactivity concentration ratios (³H-nicotine:total ³H) of parotid and submandibular saliva and of plasma in monkeys were lower than those in dogs at any time after administration. The ³H-nicotine:total ³H ratios of parotid and submandibular saliva were higher than the ratios of plasma at any time in both species. The time at which the ³H-nicotine:total ³H ratio became 0.5 (signifying that nicotine and its metabolites were in equal amounts) was [illegible].

Chromatograms of Saliva and Plasma

Chromatographic analysis was made of parotid and submandibular saliva, and plasma collected 5, 15 and 60 min after iv injection of 100 μg of ³H-nicotine. Typical chromatograms of parotid saliva in both species are shown in Fig. 4. The chromatograms separate several radioactive compounds in addition to unchanged nicotine. The

TABLE 1

TIME AT WHICH ^{3}H-NICOTINE : TOTAL ^{3}H-RADIOACTIVITY RATIO BECOMES 0.5 IN DOGS AND MONKEYS FOLLOWING INTRAVENOUS INJECTION OF 100 μG/KG OF ^{3}H-NICOTINE[a]

Samples	Time (min)	
	Dog	Monkey
Plasma	4.2	2.3
Submandibular saliva	9.4	4.2
Parotid saliva	6.6	4.1

[a] These values were obtained from the same animals as those in Figs. 1 and 2.

chromatographic patterns and R_f values were essentially identical for saliva from 2 dogs and 2 monkeys. The chromatographic patterns changed with time. Two peak areas of radioactivities found in paper chromatograms of the dog and monkey parotid saliva and plasma at 5 min were developed in the acid and base solvent system: these corresponded

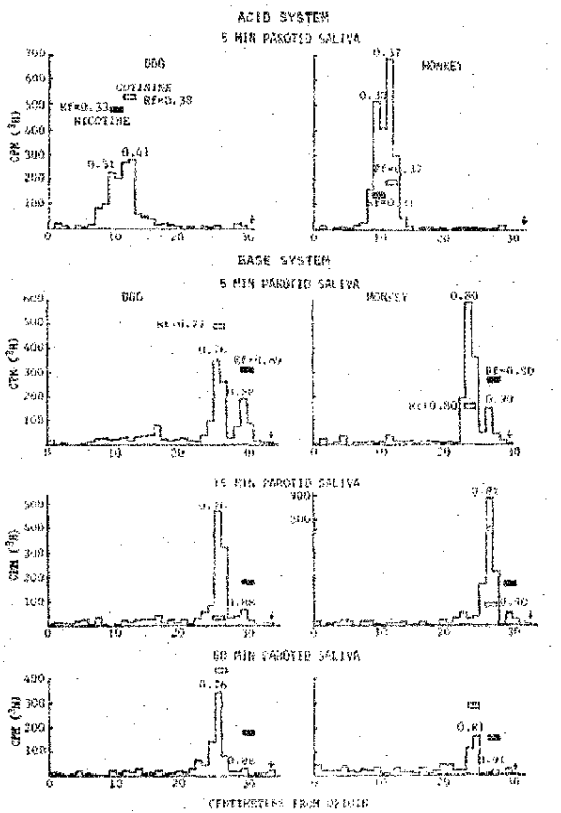

FIG. 4. Radioactivity scans of paper chromatograms of parotid saliva from a dog and a monkey 5, 15, and 60 min following iv injection of ^{3}H-nicotine (100 μg/kg). Solid and open block bars represent the location of dye-colored spots of authentic nicotine and cotinine, respectively, used as standards. Numbers over radioactive peaks indicate R_f values. Arrows indicate solvent front.

in R_f values to those of authentic nicotine and cotinine. The peak areas of radioactivity corresponding in R_f to that of authentic nicotine decreased with time. The peak areas corresponding in R_f to that of authentic cotinine increased at 15 min and then reduced at 60 min. The peak areas corresponding in R_f to that of cotinine at 5 and 15 min in monkeys were higher than those in dogs, but the peak area at 60 min in monkeys was lower than that in dogs.

R_f values for radioactive areas in chromatograms of the dog parotid saliva developed in the base system were about 0.15, 0.24, 0.34, 0.40, 0.49, 0.60, 0.67, 0.76 and 0.88. The R_f values in those of monkey parotid saliva were about 0.15, 0.24, 0.31, 0.40, 0.49, 0.54, 0.60, 0.67, 0.80 and 0.90. The chromatographic data suggest the presence of as many as 8 different metabolites in dog saliva and 9 in monkey saliva. No positive identification was made since authentic samples of probable metabolites were not available. The chromatographic patterns of submandibular saliva resembled those of parotid saliva collected in the same species at the same time. In general, the chromatographic patterns of plasma, developed in the base system, resembled those of saliva in the same species at the same time except for a few points, viz., in plasma the areas of radioactivities corresponding in R_f values to those found at about 0.60 and 0.67 in saliva were not clearly separated, and in monkey plasma radioactive areas were detected at about 0.08 but not at 0.15.

DISCUSSION

At very short times (0–1 min) after nicotine injection, total radioactivities consisting mainly of unchanged nicotine appeared in the submandibular and parotid saliva of both species. The concentrations of unchanged nicotine and total radioactivities in submandibular saliva in dogs and monkeys were higher than those in parotid saliva in both species, especially in early time. These results show that nicotine and its metabolites cross submandibular epithelium into saliva more readily than parotid epithelium in both species. At early times (0–2 min), the concentrations of nicotine in parotid and submandibular saliva of dogs were higher than those in parotid and submandibular saliva of monkeys, respectively. S:P ratios for nicotine in parotid and submandibular saliva of dogs at 1.5 and 3.5 min were higher than those in both saliva of monkeys at the same time. These results show that nicotine more rapidly crosses the salivary epithelium of dogs into saliva than in monkeys.

The S:P ratios of both glands for nicotine were significantly higher than those for total radioactivities in dogs and in monkeys, except for a few points. It is possible that nicotine crosses salivary epithelium from plasma into saliva more readily than its metabolite, cotinine. This consideration may be supported by the findings of Bowman *et al.* (1964) that cotinine resided longer in the blood stream and was generally not taken up by tissues with the same speed as nicotine. Glandular epithelium consists of 2 compartments, basal and apical membrane. Borzelleca and Putney (1970), in agreement with the finding of Burgen (1956), suggested that the movement across the apical membrane is the rate-limiting lipid diffusion step and will exhibit pH dependence, and that the movement across the basal membrane, from the plasma into the epithelial cells, appears to occur by a process of diffusion through aqueous pores. The difference in rate of movement across the salivary epithelium into saliva between nicotine and cotinine may, therefore, be related to lipid solubility of the 2 compounds.

In the present experiments, variation of salivary flow rates from 100 to 1200 μl/min did not significantly change the concentrations of nicotine and total radioactivities and the S:P ratios. This is in agreement with findings for sulfonamides and barbiturates at flow rates of 40 to 2000–3000 μl/min in cow and goat (Rasmussen, 1964) and of 250 to 2100 μl/min in man (Killmann and Thaysen, 1955). In the dog parotid saliva, concentrations of nicotine and total radioactivities did not differ with varying methods of stimulation.

The chromatographic data show that the major part of administered nicotine appeared in plasma and saliva in the form of cotinine 5, 15 and 60 min after administration. Reduction of the peak area corresponding in R_f to that of cotinine at 60 min indicates further metabolism of cotinine (McKennis, 1965). In agreement with the finding of total radioactivities in saliva, the peak areas corresponding in R_f to those of cotinine in saliva at 5 and 15 min in monkeys were higher than those in dogs. The times at which ^{3}H-nicotine:total ^{3}H ratio became 0.5 in plasma and saliva were very short in both species; in addition, those in monkeys were short relative to dogs. ^{3}H-Nicotine:total ^{3}H ratios of parotid and submandibular saliva and of plasma in monkeys were lower than those in dogs. Turner (1969) reported that significant amounts of cotinine appeared in the blood 2.5 min after nicotine injection in cats. These results suggest that nicotine is destroyed extremely rapidly and that the rate of nicotine destruction in monkeys may be faster than that in dogs; this is in agreement with findings that nicotine metabolizing enzyme activities of monkey liver are higher than those of dog liver (unpublished data from experiments now in progress in this laboratory). It has been shown that cotinine and other metabolites of nicotine are devoid of, or low in, many of the distinguishing pharmacological activities associated with the parent compound nicotine (Larson and Silvette, 1968). The quantitative differences in responses to nicotine between both species may partially result from different rates of metabolism.

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